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Isolation and *in vitro* screening for moisture and salinity stress of plant growth promoting bacterial endophytes of sorghum (*Sorghum bicolor* L.)

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Abstract
Bacterial endophytes are the microorganisms residing inside the plant tissues and have been known enhance the plant growth. However little is known about the plant growth promoting activities of sorghum endophytes. Therefore, the aim of this study was to elevate the *in vitro* bacterial mechanisms related to the plant growth promotion and their tolerance for polyethylene glycol (PEG) and sodium chloride (NaCl) in culture media. Total 55 bacterial strains were studied for both stress tolerance under varying concentration of PEG and NaCl. Out of them, five bacterial strains (AUST 3, AUST 20, AUST 29 AUST 44 and AUST55) were found tolerate to stress (25% PEG 8000 and 115 NaCl) and further used for biochemical characterization of IAA, Pi-solubilization and ACC-deaminase activities along with reference isolate *P. Fluorescens*. The strain AUST 3 had highest IAA activity (15.25 µg ml^{-1} broth), the highest phosphate solubilization potential was shown by the reference isolate *P. Fluorescens* (14.73 ppm) followed by AUST 3 (14.50 ppm) and all five potent endophytes have shown positive result for ACC deaminase activity.

Keywords: Endophyte, *Rabi* sorghum, abiotic stress tolerance, plant growth promoting trait

1. Introduction
Almost all vascular plant species, including sorghum, are found to be associated with endophytic bacteria, which may produce various bioactive compounds related to the host. Sorghum (*Sorghum bicolor*) is considered as one of the most important cereal crops in some parts of the world, particularly in arid or semi-arid region. In arid and semiarid regions soil salinity and drought are a major problem because of low precipitation and high temperatures in these regions where there is no adequate rainfall to carry the salts from the root zone of the plant and the crop is mainly grown during *Rabi* season under reserved moisture condition. Concerning to minimize the detrimental effects of the conventional techniques of agriculture, innovative methods based on microbial inoculation are recently gaining more interest. They are considered as agents to stimulate plant growth by solubilizing phosphate, producing phytohormone (such as indole acetic acid), enhancing uptake minerals (Ryan *et al.*, 2008) [14].
Many literatures are replete with reports describing the plant growth promotion under abiotic stresses in the occurrence of bacterial endophytes. Several approaches have been adopted in order to minimize the adverse effect of abiotic stresses including genetically modified crop, but the use of a diverse species of microorganisms containing ACC-deaminase shown to promote plant growth is well known and sustainable approach for enhancing plant tolerance to abiotic stresses including drought, salinity and increases growth. Endophytes having many plant growth promoting (PGP) traits such as ACC-deaminase, volatiles, antioxidants, Indole-3-acetic acid (IAA) etc. maintain reactive oxygen species (ROS) level in the plants by increasing expression of antioxidant enzymes and maintaining the plant phytohormone levels such as ethylene, ABA, IAA etc. under drought and salt stress (Yang *et al.*, 2009) [18]. The earlier have been reported the beneficial effect of bacterial inoculum on the plant growth through direct and indirect mechanisms of PGPB under different environmental stressed conditions.
Here, the main focus of the present work is to study the tolerance level of bacterial endophytes and to check the ACC-deaminase activity and other plant growth promoting (PGP) activities of selected drought and salt tolerance bacterial strains.

2. Material and methods

2.1 Source of samples

The bacterial endophytes were isolated from root and stem of the one month old healthy flowering plants of sorghum and seeds were collected after harvest from the Sorghum scheme UAS, Dharwad.

2.2 Isolation of endophytic bacteria

The plant samples were washed under running tap water for 10-15 min. to remove adhering soil particles and air-dried. The separated plant roots, stems and seeds were weighed up to one gram on a weighing balance. The samples were then surface-sterilized by dipping in 70% ethanol for 1 min, 3% sodium hypochlorite for 3 min. followed by 70% ethanol wash for 1 min (Kharwar *et al*., 2008) [8]. They were then rinsed four times with sterile water and air dried in laminar flow.

These surface sterilized samples were then macerated in one ml of distilled water in pestle and mortar. The dilutions were prepared up to 10^{-5} dilutions. One hundred micro liters from each dilution of the respective sample was then poured in their respective petri plates so labeled from 10^{-1} to 10^{-5} containing nutrient agar medium (NA) and then spread with spreader for the isolation of the bacterial endophytes. The plating was done in triplicate for each dilution. The plates were incubated at 37 °C for 72-96 hrs. (Long *et al*., 2003) [10]. Sterility check was performed by imprinting the surface sterilized plant samples of roots, stems and seeds in the media.

2.3 Drought and salinity tolerance

PEG 8000 and NaCl were added into nutrient agar medium at various concentrations in the range of 5 to 30% and 1 to 12.5% respectively and the test bacterial isolates were streaked. The growth of isolates at higher concentration was observed by their O. D value in nutrient broth.

2.4 Indole acetic acid (IAA) production

IAA production in different isolates of endophytes were detected according to Gordon and Weber (1951) [5] by inoculating bacterial suspension in 10 ml nutrient broth containing L- tryptophan 5mM (L-Trp) separately and incubated at 28±2 °C @ 180 rpm for 3 days. The culture was centrifuged at 5000 rpm for 5 min. Appearance of pink colour confirmed the production of IAA. The amount of IAA (µg ml^{-1}) was determined quantitatively by adding 1ml of Salkowski's reagent (1 ml of 0.5ml $FeCl_3$ in 50 ml of 35% perchloric acid) to 1 ml of culture supernatant along with uninoculated broth with Salkowski's reagent as a reference. The mixture was incubated in dark for 30 min @ room temperature. Absorbance of pink colour was measured spectroscopically (Bio spectrophotometer Basic, Eppendrof) at 535 nm after 20 min and quantified IAA concentration by standard curve.

2.5 P solubilization

The bacterial endophytes were inoculated to 100 ml of Pikovskaya broth (Pikovskaya, 1948) [13]. The flasks were incubated at 28 °C for 15 days. After 15 days, the Pikovskaya broth cultures were centrifuged at 9000 rpm for 20 mins. The supernatant was used for the estimation of inorganic phosphorus released by cultures by molybdenum-blue method (Murphy and Riley, 1962) [12]. The amount of phosphate released by the bacterial endophytes into the flasks was estimated in comparison with a set of absolute control without any culture.

One millilitre of the supernatant of each bacterial isolate was taken in a 50 ml volumetric flask. To this, add 10 ml of chloromolybdic acid and mix thoroughly and the volume was made to three-fourth with double distilled water. Add 0.25 ml of Chlorostannous acid and volume was made up to 50 ml with double distilled water. The blue colour was developed after 15 minutes and spectrophotometer readings were taken at 610 nm. Blank readings were also taken. Standard graph was plotted using known concentrations of standard potassium dihydrogen phosphate solution (KH_2P0_4). The amount of inorganic phosphorus released into broth was calculated from the standard graph.

2.6 ACC deaminase activity

The qualitative estimation of ACC deaminase in the endophytic bacterium was done by method of Govindasamy *et al*. (2008) [6] by streaking on Dworkin and Foster (DF) medium plates (Dworkin and Foster, 1958) [3] minimal medium containing ACC as only nitrogen supply. Incubated was done at 28±2 °C for 3-4 days and observed for growth of different bacterial isolates.

3. Results

3.1 *Invitro* screening of endophytes for induced drought and salinity

The fifty-five bacterial entophytic isolates were screened for induced drought and salinity stress. Five drought and salt tolerant isolates (AUST 3, AUST 20, AUST 29, AUST 44 and AUST 55) were selected from a total of fifty-five endophytic bacterial strains isolated from various parts of the sorghum plant based on qualitative and qualitative ability to grow under PEG 8000 induced drought and NaCl induced salt stress. Five bacterial isolates showed the growth in highest concentrations of PEG 8000 (25%) and NaCl (11%) (Table 1 and 2). Bacterial isolates that grew on media containing a high concentration of PEG were considered drought tolerant, while isolates that grew on media containing a high concentration of NaCl were salt tolerant.

3.2 *Invitro* growth profile of bacterial endophytes under induced drought and salt stress conditions

All five salt tolerant and drought-tolerant strains were tested for growth potential in liquid media under PEG 8000 and salt stress conditions (*invitro)*. By 24 hrs. of incubation, the isolate AUST 3 had reached a maximum growth rate of 1.57 O.D under normal condition. The isolate AUST 3 demonstrated better drought tolerance up to 25% PEG 8000 with an O.D value of 0.73 and salt tolerance up to 11% NaCl with an O.D value of 0.56 (Table 3).

3.3 Screening of plant growth-promoting properties of endophytes

3.3.1 Quantitative estimation of IAA by the selected endophytic bacterial isolates

Indole Acetic Acid (IAA) production is one of the plant growth promoting properties of endophytic bacteria that stimulate and facilitate plant growth. The amount of IAA produced by different isolates ranged from 7.23 to 15.25 µg ml^{-1} broth (Table 4). Among the isolates tested, AUST 3 produced maximum amount of IAA (15.25 µg ml^{-1} broth),

followed by the reference isolate, *P. fluorescens* (15.20 µg ml^{-1} broth), AUST 20 (13.60µg ml^{-1} broth). The least amount of IAA production was recorded in the isolate AUST 55 (7.23 µg ml^{-1} broth).

3.3.2 Inorganic phosphate solubilization potential of endophytic bacterial isolates

The amount of phosphate released in the flasks after incubation was estimated in comparison with a set of uninoculated controls. The results are presented in Table 4. The highest phosphate solubilization potential was shown by the reference isolate *P. fluorescens* (14.73 ppm) followed by AUST 3 (14.50 ppm) and the least was recorded by AUST 55 (7.33 ppm).

3.3.3 Alpha Amino cyclopropane carboxylic acid (ACC) deaminase producing bacteria

The ability of bacterial isolates to produce alpha-Amino cyclopropane carboxylic acid (ACC) was investigated using bacterial growth on DF salt media containing 3 mM ACC. In a plate assay, the bacterial isolates were tested for their ability to use ACC as the sole nitrogen source. The results for above mentioned assays have been depicted in Table 4. All five selected bacterial endophytes have shown positive result for ACC deaminase activity.

4. Discussion

Plants are continuously exposed to abiotic stresses such as drought, salinity and cold (Bardi and Malusa, 2012) [1]. The ravaging effects of drought on plants can be reduced by the action of endophytes with PGP traits. These bacteria are capable of tolerating and surviving under harsh environments through the regulation of phytohormones, production of ACC deaminase activity, accumulation of osmolytes, production of volatile compounds and antioxidant defense (Vurukonda *et al*., 2016) [17]. Evaluation of the potential of endophytes isolated from sorghum plant as plant growth promoters were conducted in this study.

In total, 55 isolates of bacteria were successfully isolated from sorghum plant and they used for preliminary screening of drought and salinity. Endophytic bacteria are particularly tolerant of environmental stresses such as high salinity, heat and cold. Our results showed that the five endophytic bacterial isolates (AUST 3, AUST 20, AUST 29, AUST 44 and AUST 55) tolerated 25% PEG and11% of NaCl. The endophytic bacterial strains of *Curcuma longa* L showed tolerance to the increasing salt concentration. *B. thuringiensis* (ECL2) and *B. pumilus* (ECL4) tolerated higher salt level (8% NaCl) whereas *B. cereus* ECL1 and *Bacillus* sp. ECL3 tolerated 7% of NaCl (Kumar *et al*., 2016) [9]. Bacterial adaptation to high drought and salt concentrations is primarily due to intracellular sodium ion balance maintenance, osmolyte secretion and changes in cell physiology to external environmental conditions.

Several endophytic bacteria interact positively, via. Various mechanisms with their host plant. They produce plant growth, phosphate solubilization, IAA production, siderophore production and activities of enzymes. IAA production and P-solubilization are found to be the most comment PGP trait reported from endophytes isolated from plants exposed to abiotic stresses, suggesting selection for such beneficial endophytes in stress conditions (Forchetti *et al*., 2007) [4]. In the present study, it was observed that several bacterial isolates isolated from sorghum plant were able to produce IAA, solubilize phosphate and have ACC deaminase acitivity (Table 4). Mohamad *et al*. (2020) [11] reported *Bacillus* sp. and *Pseudomonas* sp. with plant growth promotion and their activity was associated with the production of IAA and siderophore. The role of ACC-deaminase in reducing stress ethylene levels via enzymatic hydrolysis of ACC into -ketobutyrate and ammonia has been proposed as one of the critical mechanisms of PGPB in promoting plant growth under extreme environmental conditions.

Table 1: *In vitro* drought (PEG 8000) tolerance of bacterial endophytes

Isolates	5%	10%	15%	20%	25%	30%	Isolates	5%	10%	15%	20%	25%	30%
AUST 1	+	+	+	+	-	-	AUST 29	+	+	+	+	+	-
AUST 2	+	+	+	+	-	-	AUST 30	+	+	+	+	-	-
AUST 3	+	+	+	+	+	-	AUST 31	+	+	+	-	-	-
AUST 4	+	+	+	+	-	-	AUST 32	+	+	+	+	-	-
AUST 5	+	+	+	-	-	-	AUST 33	+	+	-	-	-	-
AUST 6	+	+	+	+	-	-	AUST 34	+	+	+	-	-	-
AUST 7	+	+	+	+	+	-	AUST 35	+	+	+	+	+	-
AUST 8	+	+	+	+	-	-	AUST 36	+	+	+	-	-	-
AUST 9	+	+	+	-	-	-	AUST 37	+	+	-	-	-	-
AUST 10	+	+	+	+	-	-	AUST 38	+	+	+	-	-	-
AUST 11	+	+	-	-	-	-	AUST 39	+	+	+	+	-	-
AUST 12	+	+	+	-	-	-	AUST 40	+	+	+	+	-	-
AUST 13	+	+	+	+	+	-	AUST 41	+	+	+	+	-	-
AUST 14	+	+	+	+	-	-	AUST 42	+	+	+	-	-	-
AUST 15	+	+	+	+	-	-	AUST 43	+	+	+	+	-	-
AUST 16	+	+	-	-	-	-	AUST 44	+	+	+	+	+	-
AUST 17	+	+	+	+	-	-	AUST 45	+	+	+	+	-	-
AUST 18	+	+	+	+	-	-	AUST 46	+	+	+	-	-	-
AUST 19	+	+	+	+	-	-	AUST 47	+	+	+	+	-	-
AUST 20	+	+	+	+	+	-	AUST 48	+	+	-	-	-	-
AUST 21	+	+	+	+	-	-	AUST 49	+	+	+	+	-	-
AUST 22	+	+	+	+	-	-	AUST 50	+	+	+	+	+	-
AUST 23	+	+	-	-	-	-	AUST 51	+	+	+	+	-	-
AUST 24	+	+	+	+	-	-	AUST 52	+	+	-	-	-	-

AUST 25	+	+	-	-	-	-	AUST 53	+	+	+	+	-	-
AUST 26	+	+	+	+	-	-	AUST 54	+	+	+	+	-	-
AUST 27	+	+	+	+	-	-	AUST 55	+	+	+	-	+	-
AUST 28	+	+	+	-	-	-							

Table 2: *In vitro* salinity (NaCl) tolerance of bacterial endophytes

Isolates	2.5%	5%	7.5%	10%	11%	12.5%	Isolates	2.5%	5%	7.5%	10%	11%	12.5%
AUST 1	+	+	+	+	-	-	AUST 29	+	+	+	+	+	-
AUST 2	+	+	+	+	-	-	AUST 30	+	+	+	+	-	-
AUST 3	+	+	+	+	+	-	AUST 31	+	+	+	-	-	-
AUST 4	+	+	+	+	-	-	AUST 32	+	+	+	+	+	-
AUST 5	+	+	+	-	-	-	AUST 33	+	+	-	-	-	-
AUST 6	+	+	+	+	-	-	AUST 34	+	+	+	-	-	-
AUST 7	+	+	+	+	+	-	AUST 35	+	+	+	+	-	-
AUST 8	+	+	+	+	-	-	AUST 36	+	+	+	-	-	-
AUST 9	+	+	+	-	-	-	AUST 37	+	+	-	-	-	-
AUST 10	+	+	+	+	-	-	AUST 38	+	+	+	-	-	-
AUST 11	+	+	-	-	-	-	AUST 39	+	+	+	+	-	-
AUST 12	+	+	+	-	-	-	AUST 40	+	+	+	+	-	-
AUST 13	+	+	+	+	+	-	AUST 41	+	+	+	+	-	-
AUST 14	+	+	+	+	-	-	AUST 42	+	+	+	-	-	-
AUST 15	+	+	+	+	-	-	AUST 43	+	+	+	+	-	-
AUST 16	+	+	-	-	-	-	AUST 44	+	+	+	+	+	-
AUST 17	+	+	+	+	-	-	AUST 45	+	+	+	+	-	-
AUST 18	+	+	+	+	-	-	AUST 46	+	+	+	-	-	-
AUST 19	+	+	+	+	-	-	AUST 47	+	+	+	+	-	-
AUST 20	+	+	+	+	+	-	AUST 48	+	+	-	-	-	-
AUST 21	+	+	+	+	-	-	AUST 49	+	+	+	+	-	-
AUST 22	+	+	+	+	-	-	AUST 50	+	+	+	+	-	-
AUST 23	+	+	-	-	-	-	AUST 51	+	+	+	+	-	-
AUST 24	+	+	+	+	-	-	AUST 52	+	+	-	-	-	-
AUST 25	+	+	-	-	-	-	AUST 53	+	+	+	+	-	-
AUST 26	+	+	+	+	+	-	AUST 54	+	+	+	+	-	-
AUST 27	+	+	+	+	-	-	AUST 55	+	+	+	+	+	-
AUST 28	+	+	+	-	-	-							

Table 3: Effect of osmotic and salinity stress on the growth of the endophytic bacteria

Isolates	Growth obtained (in O.D)		
	Control	25% PEG	12.5% NaCl
AUST 3	1.57	0.73	0.56
AUST 20	1.25	0.70	0.46
AUST 29	0.69	0.32	0.21
AUST 44	0.95	0.51	0.30
AUST 55	0.75	0.40	0.21
Pseudomonas fluorescens	1.54	0.68	0.53

Table 4: Plant growth promoting traits of the bacterial endophytes

Isolates	IAA (µg/ml broth)	P solubilization (ppm)	ACC deaminase activity
AUST 3	15.25	14.50	+
AUST 20	13.60	11.32	+
AUST 29	8.05	8.44	+
AUST 44	11.05	9.56	+
AUST 55	7.23	7.33	+
Pseudomonas fluorescens	15.20	14.73	+

5. Conclusion

Total fifty five bacterial endophytes screened for *invitro* drought and salinity. Out of these five endophytic bacterial isolates (AUST 3, AUST 20, AUST 29, AUST 44 and AUST 55) tolerated high drought (25%) and salt (11% of NaCl) concentration. These bacterial isolates were able to solubilize phosphate IAA and ACC deaminase activity. The higher IAA activity was recorded in AUST 3 followed by reference isolate *P. fluorescens* and higher Pi-solubilization was noticed in reference isolate followed by AUST 3. All the five isolates have shown positive result for ACC deaminase activity. This study provides future encouragement for the plant growth promoting endophytic bacterial isolates for the improvement of plant growth under drought and salinity stress conditions.

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